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ORIGINAL PAPER

Macrophage migration inhibitory factor (MIF) of the protozoan parasite *Eimeria* influences the components of the immune system of its host, the chicken

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Abstract Macrophage migration inhibitory factor (MIF) is a soluble factor produced by sensitized T lymphocytes that inhibits the random migration of macrophages. Homologues of MIF from invertebrates have been identified, making it an interesting molecule from a functional perspective. In the present study, the localization of a parasite MIF protein as well as its effect on the host was characterized. Western blot analysis shows that *Eimeria* MIF (EMIF) is found during all parasite developmental stages tested. Transmission electron microscopy shows that MIF is distributed throughout cytosol and nucleus of *Eimeria acervulina* merozoites. Immunohistochemical analysis suggests that EMIF may be released into the surrounding tissues as early as 24 h after infection, while later during oocyst formation, MIF expression is localized to areas immediately surrounding the oocysts, as well as in wall-forming bodies. The chemotaxis assay revealed an inhibitory function of EMIF on chicken monocyte migration. Quantitative real-time PCR was performed to examine the effect of EMIF on host immune system by measuring the transcripts of inflammatory mediators. An ex vivo stimulation study showed that *E. acervulina* MIF (EaMIF) enhanced expression of pro-inflammatory cytokines and chemokines in the presence of lipopolysaccharide (LPS). Furthermore, sequential treatment of adherent peripheral blood mononuclear cells with EaMIF, chicken MIF, and LPS in 2-h intervals led to the highest levels of interleukin (IL)-1B, chemokine CCLi3, IL-18, and interferon-gamma mRNA expression. This study shows that parasite MIF is widely expressed and may have potential effects on the immune system of the host.

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Introduction

Macrophage migration inhibitory factor (MIF) was originally identified as a soluble factor produced by activated T cells that inhibited the random migration of macrophages (Bloom and Bennett 1966; David 1966). However, a biologically active recombinant clone of human MIF was not produced for over 20 years following the initial identification of the soluble factor (Weiser et al. 1989). Recent studies revealed an essential role of MIF in adaptive immune responses as well as innate immunity. For instance, mammalian MIF is an immunomodulator that controls macrophage function, resulting in the promotion of pro-inflammatory cytokine expression including tumor necrosis factor-alpha (TNF-α), interleukin (IL)-1B, IL-2, IL-6, IL-8, and interferon-gamma (IFNG) (Calandra et al. 1994; Bacher et al. 1996; Donnelly et al. 1997), nitric oxide (NO) release (Bernhagen et al. 1994), and cyclooxygenase 2 (COX-2) activity (Mitchell et al. 2002). Macrophage migration inhibitory factor also upregulates the expression of Toll receptor 4, which encodes the signal-transducing element of the LPS receptor

complex and thus plays important role in early innate immunity inducing the activation of monocytes and macrophages (Roger et al. 2001). For its role in adaptive immunity, MIF is constitutively expressed by T lymphocytes but can also be induced by mitogens, CD3-specific antibody, and glucocorticoids (Bacher et al. 1996; Calandra et al. 1998; Abe et al. 2001). Produced primarily by activated T helper 2 (Th2) cells, MIF appears to have a possible autocrine function, resulting in activation and proliferation of T cells and IL-2 production (Bacher et al. 1996). Moreover, MIF inhibits regulatory effects on cytotoxic $CD8^+$ T cells and regulates lymphocyte trafficking (Abe et al. 2001).

Characterization of MIF in nonmammalian species is limited. Recently, some of the biological properties of chicken MIF (ChMIF) have been reported (Kim et al. 2010). It was found that ChMIF alone does not promote the expression of pro-inflammatory cytokines and NO release (Kim et al. 2010). However, ChMIF induces the expression of mRNA of pro-inflammatory cytokines (IL-1B, IL-6, IL-8, and IL-12), as well as NO, when chicken peripheral blood mononuclear cells (PBMCs) are initially primed with LPS. ChMIF also enhances Th1/Th2 cytokines in previously stimulated lymphocytes (Kim et al. 2010). In a recent study, Jang et al. 2011 reported that stimulating a transformed chicken macrophage cell line, HD11, with recombinant (rChMIF) resulted in upregulation of transcripts encoding IL-6, IL-17, and tumor necrosis factor superfamily member 15 but decreased the expression of IL-8. Both studies observed effects of rChMIF on chicken cells but with disparate findings. Kim et al. (2010) observed an almost complete inhibition of migration of PBMCs, while Jang et al. (2011) reported that rChMIF increased the chemotaxis of chicken macrophages.

Miska and colleagues (2007) characterized a MIF homologue from the apicomlexan parasites *Eimeria acervulina* and *Eimeria tenella*. Both of these organisms cause coccidiosis in chickens. Coccidiosis results in an estimated loss of $3 billion annually worldwide (Dalloul and Lillehoj 2006). Seven species have been recognized to infect the chicken host: *E. acervulina*, *Eimeria brunetti*, *Eimeria maxima*, *Eimeria mitis*, *Eimeria necatrix*, *Eimeria praecox*, and *E. tenella* (Allen and Fetterer 2002; McDonald and Shirley 2009). However, *E. maxima*, *E acervulina*, and *E. tenella* are often considered to be the most pathogenic species in broiler chickens. Infections (usually a mix of species) occur due to ingestion of oocysts and lead to digestive disorders resulting from damage to the intestinal epithelium, malabsorption of nutrients, changes in protein metabolism after absorption, reduced feed conversion efficiency, and reduction in weight gain (Conway et al. 1993; Shirley et al. 2005). The disruption of the intestinal epithelial layer leads to the diminished ability of the intestine to absorb nutrients, resulting in reduced performance and higher susceptibility to other diseases such as necrotic enteritis (Yegani and Korver 2008).

It is not clear what biological roles MIF may play in *Eimeria* or in the reaction of *Eimeria* with its host. An initial characterization of MIF from *E. acervulina* (EaMIF) and *E. tenella* (EtMIF) determined that MIF is developmentally regulated, is located in the cytosol with greater concentrations associated with the apical end of the merozoites, and can be excreted (Miska et al. 2007). In the present study, the biological activity of *Eimeria* MIF (EMIF) is further described, and localization of EtMIF using immunohistochemistry and immunoelectron microscopy is performed.

Materials and methods

Parasites

Oocysts of *E. tenella* (Wampler strain) and *E. acervulina* (strain 12) were maintained by passage through 2–3-week-old chicks as previously described (Fetterer and Barfield 2003). All birds were housed and reared according to the Institutional Animal Care and Use Committee (IACUC) of Beltsville Area Research Center. For sporulation time-course studies, unsporulated oocysts (UO) collected from feces of infected birds were incubated with sodium hypochlorite (6 %) on ice for 10 min in twice the volume of the pellet with agitation every 2–3 min. Following treatment with 5 % bleach, samples were diluted with water, and the bleach was removed by repeated washing with water followed by centrifugation. Oocysts were suspended in PBS containing an antibiotic/antimycotic mixture (1,000 μg/ml penicillin; 1,000 μg/ml streptomycin; 25 ng/ml fungizone; Life Technologies) and incubated under aeration at 41 °C. At the desired time interval (ranging from 0 to 72 h), an aliquot containing about 1×10^8 oocysts was removed from the incubation flask and centrifuged, and the pellet containing oocysts was resuspended in 1.0 ml of 40 mM Tris and stored at −70 °C.

Sporozoites (SZ) were prepared from sporulated oocysts (less than 30 days post-harvest) as previously described (Fetterer and Barfield 2003) and were purified by filtering through a cellulose filter pad (Fetterer et al. 2004; Fuller and McDougald 2001). Isolated SZ were frozen at −70 °C. For merozoites (MZ) collection, 10–20 broiler chicks (2–4 weeks of age) were inoculated with 400,000–500,000 sporulated oocysts (SO), and sacrificed at 89 h (*E. acervulina*) or 110 h (*E. tenella*) post-infection (PI). About 15 cm of the duodenum or both ceca were removed, and MZ was isolated as previously described (Schwarz et al. 2010). By microscopic examination, the preparation contained primarily merozoites with some schizonts. Following isolation, the merozoites were pelleted and snap-frozen at −70 °C.

Expression and purification of recombinant MIF

Previously described full-length EaMIF and EtMIF cDNA clones (Miska et al. 2007) served as a template for amplification

of cDNAs for production of expression constructs. Primers for amplification of EtMIF included the *Nde*I and *Bam*HI restriction sites, respectively, (KM198-F 5′ GTCATATGCCACT GTGCCAGATC 3′, KM199-R 5′ CAG**GATCC**TTA ACCAAACACGCG 3′). Primers for amplification of EaMIF included the sequence for *Nhe*I and *Bam*HI sites, respectively, (KM171-F 5′ ATG**CTAGC**CCGCTCTGCCAGATC 3′, KM172-R 5′ ATG**GATCC**GGCGAAAACGCGAGACC 3′). The EtMIF cDNA was digested with *Nde*I and *Bam*HI (NEB), while EaMIF cDNA was digested NheI and BamHI (NEB). Digested cDNA was gel-purified using QIAspin columns (Qiagen) and ligated into *Nde*-*Bam*HI or *Nhe*I-*Bam*HI digested pET11(a) vector (EMD Biosciences) and transformed into *E. coli* TOP10 cells (Invitrogen). Maintenance of the correct reading frame was confirmed by sequencing positive transformants in both directions with the T7 promoter (5′ TAATACGACTCACTATAGGG 3′) and the T7 terminator (5′ GCTAGTTATTGCTCAGCGG 3′) primers (EMD Biosciences). Positive clones were then transformed into *E. coli* BL21 cells (EMD Biosciences). Construction of the rChMIF clone has been previously described by Kim et al. (2010).

E. coli BL21 cells (EMD Biosciences) containing recombinant EtMIF-pET11 or EaMIF-pET11 were cultured in LB medium containing 100 μg/ml ampicillin in an orbital shaking incubator at 37 °C until mid-log phase (OD600=0.5). Recombinant EtMIF and EaMIF protein production was induced by incubating the culture for an additional 4 h in the presence of 1 μM isopropyl thiogalactopyranoside. The cultures were harvested by centrifugation at 25,000×*g* for 10 min, followed by resuspension of the pellet in 20 mM NaPO4, 500 mM NaCl (pH7.8) buffer. The cells were lysed by three freeze–thaw cycles between dry ice–ethanol bath and a 37 °C water bath, followed by treatment with 1 U/ml DNase and RNase (Sigma) for 30 min at room temperature (RT). The soluble fraction containing rEtMIF or rEaMIF was recovered by centrifugation of the extract at 5,000×*g* for 15 min.

To reduce endotoxin levels, the bacterial lysate containing rEtMIF or rEaMIF was extracted with octylphenoxypolyethoxyethanol (TX-114; Sigma) prior to purification by using a slight modification of a previously described procedure (Liu et al. 1997). Following TX-114 extraction, rMIF was purified from the extract by size-exclusion high-performance liquid chromatography (SEC–HPLC) as previously described (Kim et al. 2010). Fractions from SEC–HPLC containing rMIF were pooled and concentrated by centrifugal filtration (Millipore). Protein concentrations were determined by the BCA assay (Thermo Scientific). To further reduce endotoxin levels, HPLC-purified rMIF samples used for cellular assays were extracted with polymyxin B bound to 45-μm beads (Affi-Prep) as described (Kim et al. 2010). Samples were reanalyzed for protein concentration, and purity was determined by electrophoresis. Protein recovery was estimated to be about 90 %. Endotoxin concentrations of samples were determined by the Limulus amebocyte assay (Lonza) performed with the microplate method following the manufacturer's recommendation.

Antibody production

Antisera to rEtMIF were made by immunizing rabbits with purified rEtMIF as previously described (Fetterer and Barfield 2003). Antisera production was performed by Pacific Immunology against rEaMIF by immunizing two rabbits with purified rEaMIF (Miska et al. 2007). Briefly, pre-immune sera were obtained from both rabbits, which were then immunized four times with 200-μg protein each. The primary immunization was done with complete Freund's adjuvant, while booster immunizations, in incomplete Freund's adjuvant. Following immunizations, the sera were collected from rabbits and frozen (−70 °C) in 1-ml aliquots.

Antibody localization

For indirect immunohistochemical (IHC) analysis of cecal tissues infected with *E. tenella*, 4-week-old chickens were infected with 150,000 sporulated *E. tenella* oocysts as previously described (Fetterer and Barfield 2003). All birds had been housed and reared according to the IACUC of Beltsville Area Research Center. Following euthanasia by cervical dislocation, approximately 1–1.5 in. of ceca was collected at 24-h intervals from infected chickens as well as time-matched controls beginning 24 h PI and continuing to 144 h PI. Tissues were fixed in 10 % formalin, and 10-μm paraffin sections were prepared (Histoserve Inc.). Staining was done using the EnVision + System-HRP (AEC) for use with rabbit primary antibodies using the manufacturer's recommended protocol (Dako). Polyclonal rabbit anti-rEtMIF as well as control sera were used at a 1:500 dilution. Stained slides were viewed and photographed using Zeiss Axioskop I microscope, and images were processed with AxioCam digital camera (Carl Zeiss Imaging).

For electron microscopy, *E. acervulina* merozoites MZ were isolated as previously described (Schwarz et al. 2010) and were fixed for 5 min in 0.5 % gluteraldehyde in 0.1 M cacodylate buffer and then washed twice with 0.1 M cacodylate buffer. After fixation, MZ were pelleted by centrifugation, and the pellet was gently washed twice with 100 μl cacodylate buffer. The pellet was dehydrated in a graded ethanol series, infiltrated overnight in LR White hard-grade acrylic resin (London Resin Company), and cured at 55 °C for 24 h. Thin sections (90-μm thick) were obtained using a Diatome diamond knife on a Reichert/AO Ultracut microtome and collected on 200-mesh Formvar-

coated nickel grids. The grids were floated with the tissue section facing down on drops of PBS containing 0.1 M glycine and 1 % BSA for 10 min, washed with PBS, floated on drops of PBS–2 % nonfat dry milk (NFDM)–0.1 % Tween 20 (Tw20), and then floated on drops containing a 1:250 or 1:500 dilution of rabbit antiserum in PBS–NFDM–Tw20. Rabbit antiserum to rEaMIF was used in the primary labeling step. The grids were incubated for 2 h at RT, washed three times with PBS–NFDM–Tw20, and floated for 1 h at RT on drops of a 1:1,000 dilution of gold particle (10-nm diameter)–labeled goat anti-rabbit IgG (H–L chain specific, Sigma). The grids were washed three times with PBS–Tw20, washed twice with deionized water, air-dried, stained with 5 % uranyl acetate for 30 min, and examined with a Hitachi H7000 electron microscope.

Western blots

Soluble extracts of *E. tenella* UO, sporulating and SO, SZ, and MZ were prepared in 40 mM Tris, pH 8.0 containing a cocktail of protease inhibitors (Complete, Roche Diagnostics) and DNase/RNAase (Sigma) as previously described (Fetterer et al. 2004). Samples were incubated for 10 min at 37 °C and then frozen at −70 °C. Concentrations of soluble proteins were measured by BCA assay (Pierce) with bovine serum albumin as the standard. Samples were analyzed by polyacrylamide gel electrophoresis using 1-mm-thick gradient gels (8–9 cm, 4–12 % Bis–Tris, Invitrogen) as described (Fetterer and Barfield. 2003). All samples were reduced by addition of dithiothreitol. For extracts of *E. tenella*, about 4 μg of protein was loaded per lane. Between 10 and 100 ng of rMIF (chicken, *E. acervulina*, or *E. tenella*) was loaded per lane. Western blot analysis was performed using the method previously described (Fetterer and Barfield 2003). Rabbit primary antibody against EaMIF and ChMIF was used at 1:1,000 dilution, while rabbit anti-EtMIF was used at 1:500 dilution. Secondary antibody was goat anti-rabbit conjugated to HRP (20 ng/ml; Pierce, IL). Chemiluminescence of blots was visualized with a digital camera after exposure to Luminol (Super Signal West Dura Extend, Pierce). Western blots were quantified with a gel analysis system (Labworks, UVP).

Chemotaxis assay using modified Boyden chamber

Adherent PBMCs were isolated from 20-week-old broiler chickens (donated by Dr. Paul Siegel, Virginia Tech) and cultured as described by Kim et al. (2010). Nonadherent cells were removed from PBMCs after 24 h in culture (Kim et al. 2010). All birds had been housed and reared according to IACUC of Virginia Tech.

The purified rEMIF was prepared by 10-fold serial dilutions (0.001 to 1 μg/ml) with DMEM supplemented with 2 mM L-glutamine and 10 % FCS. The medium supplemented with 10 % FCS was used as positive control to induce cell migration, while serum-free medium was used as negative control. Recombinant protein and controls (25 μL) were pipetted into the bottom wells of the chamber, separated from the top wells by 5-μm pore of polycarbonate filter membrane. Isolated adherent PBMCs were adjusted to 1×10^6 cells/mL, and 50 μl of suspended cells was loaded into the top wells of the chamber. Migration was allowed to continue for 4 h at 39 °C in humidified air containing 5 % CO_2. The cells at the lower surface of the membrane were fixed and stained with Diff-Quick Staining (Fisher Scientific). The stained cells were counted, and the percentage of migration inhibition was determined by applying the following formula (Weiser et al. 1989; Jin et al. 2007):

$$\text{Percent migration inhibition} = \left(1 - \frac{\text{Mean area of migration in experiment group}}{\text{Mean area of migration in control group}}\right) \times 100$$

Ex vivo cell stimulation for biological function analysis

The isolated adherent PBMCs (described above) were seeded onto 12-well plates and cultured for 24 h. After the cells were gently washed, they were treated for 6 h in two different groups as shown in Table 1. Cell lysis buffer was directly added into the wells and total RNA was extracted using RNeasy Mini Kit (Qiagen). Using 1 μg of total RNA, the first-strand cDNA was synthesized using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems) following the manufacturer's instruction (Kim et al. 2010), and the cDNA was stored at −20 °C.

Quantitative real-time PCR analysis of the cytokine transcripts

To analyze the transcripts of various cytokines, primers were designed using Primer Express (Ver 3.0; Applied Biosystems) (Table 2), and quantitative real-time (qRT)-PCR was performed using Fast SYBR Green Master Mix (Applied Biosystems) as described by Kim et al. (2010). Target gene expression in this study was normalized against the expression of chicken GAPDH mRNA, followed by analysis of the results using ABI 7500 Software (Ver 2.0; Applied Biosystems).

Statistical analysis

All data were analyzed by either Student's *t* test or analysis of variance using JMP (Ver 8.0) software, and significant differences among groups were tested by the Tukey–Kramer honestly significant difference post hoc procedure.

Table 1 Treatment of chicken PBMCs with ChMIF, EaMIF, and LPS

Treatment (μg/mL)	Group 1							Group 2				
ChMIF (0.01)	−	+	−	−	+	−	+	+	−	+	+	+[a]
EaMIF (0.01)	−	−	+	−	+	+	+	+[a]	+	+	+[a]	+
LPS (5)	−	−	−	+	−	+	+	−	+[a]	+[a]	+[b]	+[b]

[a] Indicates that the marked reagent was added 2 h later the first treatment ("+") was added

[b] Indicates that the marked reagent was added 2 h later "a" reagent was added

Results

Developmental expression and localization of *Eimeria* MIF

Western blot analysis indicates that EtMIF is present in several stages of the parasite's life cycle. Presence of MIF was observed in UO, sporulating oocysts, SO, as well as MZ and SZ (Fig. 1). Immunoelectron microscopy was performed on *E. acervulina* MZ in order to determine the localization of MIF inside the parasite (Fig. 2). The cytosol of *E. acervulina* MZ reacts with the antisera to EaMIF and appears scattered throughout the cytosol, and staining was not associated specifically with any structure or organelle. However, a significant amount of staining was also observed in the nucleus (Fig. 2a–c). No staining was seen when pre-bleed control antiserum is used instead of the primary antibody (Fig. 2d).

After 24 h PI, the columnar epithelial cells lining the villi of the ceca were positively stained when incubated with rabbit anti-EtMIF polyclonal antisera (Fig. 3a). A similar pattern of staining was observed at 48 h PI (data not shown). No staining was observed when normal rabbit sera were used in place of the primary antibody (Fig. 3b) nor when ceca from normal, uninfected, chickens were stained with anti-EtMIF (Fig. 3c). At 96 h PI, staining of schizonts was observed (Fig. 3d). Staining of macrogametes and developing oocyts was observed at 120 h PI (Fig. 3e), and the wall-forming bodies of the oocysts as well as the outer edges of the oocysts stained positively against EtMIF. Sections stained with control rabbit sera did not produce any staining (Fig. 3f).

In order to confirm that the primary antibody against EtMIF did not cross-react with ChMIF, we tested the cross-reactivity of rabbit anti-rEtMIF using Western blots. The antibody against rEtMIF did not cross-react against purified rChMIF (Fig. 4a). Similarly, the antibody against rEaMIF did not cross-react with rChMIF but did bind to rEtMIF (Fig. 4b).

Effect of rEaMIF and rEtMIF on migration of chicken monocytes

To examine the effect of rEaMIF and rEtMIF on chemotaxis of chicken monocytes, isolated chicken adherent PBMCs were incubated with different concentrations of rEaMIF or rEtMIF (Fig. 5). Both rEaMIF and rEtMIF showed inhibitory function of macrophage migration. The lowest concentration (0.001 μg/ml) of rEaMIF tested in this study showed approximately 60 % inhibition of chicken monocyte migration, and the highest concentration (1 μg/ml) showed approximately 90 % inhibition. Percentage of inhibition by rEtMIF exhibited a very similar pattern to that of EaMIF, except EtMIF showed approximately 78 % inhibition of chicken monocyte migration at the lowest concentration.

The effect of *Eimeria* MIF on adherent PBMC function

To determine the effect of EMIF on chicken monocyte function, freshly cultured chicken PBMCs were incubated with different combinations of rEaMIF, rChMIF, and LPS as shown in Table 1. As previously found with rChMIF (Kim et al. 2010), rEaMIF alone had no effect on the expression of IL-1B, IL-18, IFNG, and the chemokine CCLi3 (Fig. 6). As expected, LPS induced expression of IL-1B, IFNG, and CCLi3 (Fig. 6). Chicken PBMCs were not influenced by mixed treatment of rChMIF and rEaMIF. However, incubation of PBMCs with rEaMIF and LPS together led to an

Table 2 Primer sequences for qRT-PCR analyses of cytokine transcripts

Primer name	Accession no.	Sense sequence	Anti-sense sequence
GAPDH	NM_204305	AGGGTGGTGCTAAGCGTGTTA	TCTCATGGTTGACACCCATCA
IFNG	NM_205149	GCTCCCGATGAACGACTTGA	TGTAAGATGCTGAAGAGTTCATTCG
IL-1B	NM_204524	CCCGCCTTCCGCTACA	CACGAAGCACTTCTGGTTGATG
IL-18	NM_204608	AGGTGAAATCTGGCAGTGGAAT	TGAAGGCGCGGTGGTTT
CCLi3	Y18692	CCTGCTGCACCACTTACATAACA	TGCTGTAGTGCCTCTGGATGA

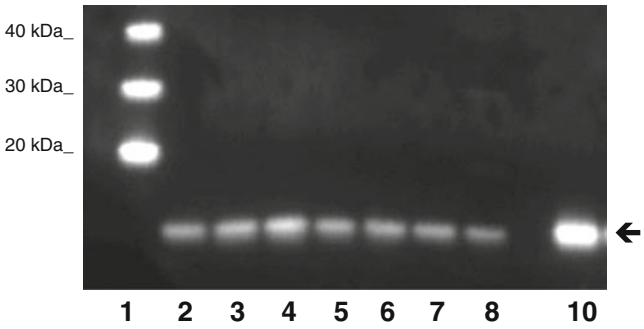

Fig. 1 Immunolocalization of EtMIF in several stages of parasite development using Western blot analysis. Parasite extracts loaded at 4 μg/lane; rEtMIF loaded at 100 ng/lane. Primary antibody used was rabbit anti-EtMIF (1:500). Secondary antibody used was goat anti-rabbit HRP 10 ng. *Lane 1*, molecular weight standards; *lane 2*, UO; *lane 3*, oocysts after 12 h of sporulation; *lane 4*, oocysts after 24 h of sporulation; *lane 5*, oocysts after 48 h of sporulation; *lane 6*, oocysts after 72 h of sporualtion; *lane 7*, SZ; *lane 8*, MZ; *lane 10*, rEtMIF

approximately 1.4-fold increase of mRNA levels of IL-1B and CCLi3 than with LPS alone (Fig. 6a, b). IFNG expression was enhanced 2.7-fold when PBMCs were incubated with rChMIF, rEaMIF, and LPS compared to only rEaMIF and LPS (Fig. 6d). A 2 h pre-treatment of PBMCs with rEaMIF, followed by incubation with LPS, resulted in 1.6-fold increase in expression of IL-1B and CCLi3. Expression of IFNG in the same 2 h pre-treatment was enhanced 5.6-fold compared to that of rEaMIF and LPS treatment. Addition of LPS in the presence of rChMIF and rEaMIF showed significantly enhanced expression of IL-1B, CCLi3, and IFNG in comparison with those treated with LPS alone or rEaMIF with LPS. A 2-h interval sequential treatment of PBMCs with rChMIF followed by addition of rEaMIF did not affect the tested immune mediators; however, sequential addition of rChMIF, rEaMIF, and LPS greatly enhanced IL-1B, CCLi3, and IFNG mRNA levels. Expression of ChIL-18 was only affected when cells were treated with rChMIF, rEaMIF, and LPS sequentially in a 2-h interval (Fig. 6c).

Discussion

The current study presents a further analysis of the MIF of *Eimeria* species that are infectious to chickens and cause coccidiosis. MIF in vertebrates is a cytokine; therefore, it is logical that, in *Eimeria*, the MIF protein may also retain some immunomodulatory functions. A previous study (Miska et al. 2007) showed that EMIF shares over 50 % sequence identity in a domain that is believed to be important for immunomodulatory activity of mammalian MIFs (Kleemann et al. 2000). Homologues from other parasites exhibit cytokine-like properties and are thought to be released from the parasite in order to modulate host immune responses (Tan et al. 2001). These observations have lead to the hypothesis that parasite MIF is key in adapting the parasite to the host. Alternatively or in addition, MIF may also play a vital role in parasite development.

Supporting a role for MIF in the parasite's biology, EtMIF protein is present in the life cycle stages examined and is located in the cytosol as well as the nucleus of the invasive merozoite. This is in contrast with a previous observation that indicated MIF in *E. acervulina* was developmentally regulated being most highly expressed in the MZ (Miska et al. 2007),

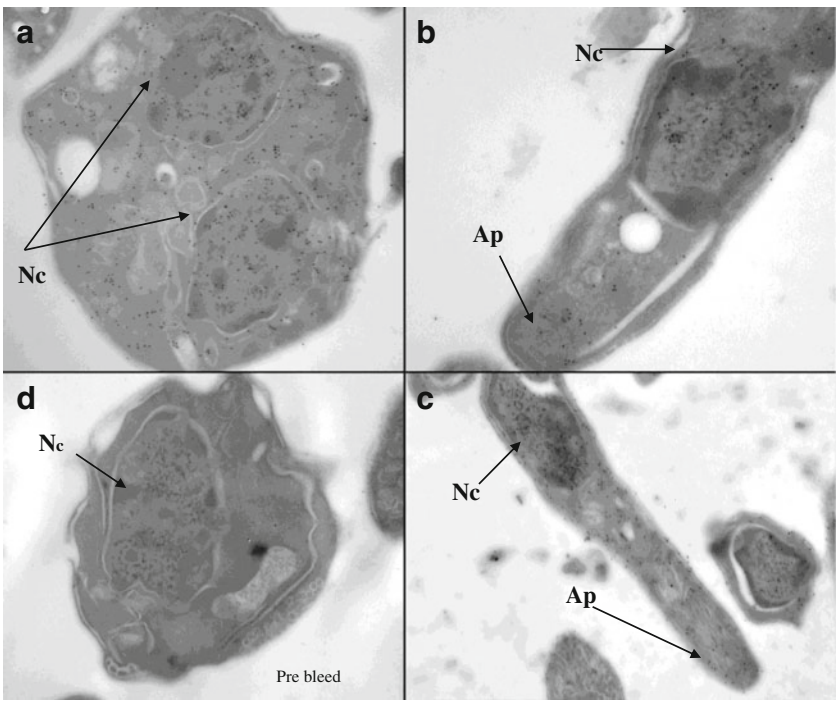

Fig. 2 Immunolocalization of EaMIF in merozoites using transmission electron microscopy. **a**–**c** Primary rabbit anti EaMIF antibody at 1:250 or 1:500 was used. **d** Pre-bleed rabbit serum was used in place of primary antibody. Secondary antibody used is gold particle-labeled goat anti-rabbit IgG 1:1,000. *Nc*, nucleus; *Ap* apical complex

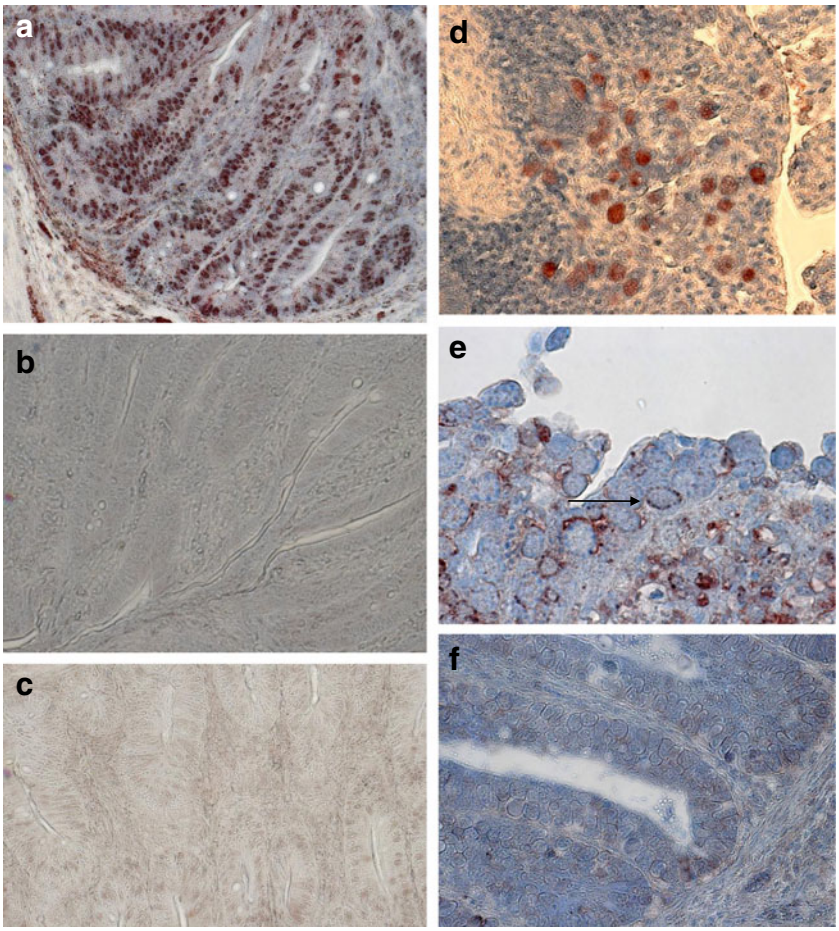

Fig. 3 Immunohistochemistry of normal uninfected (**c**) or Et-infected (**a**, **b**, **d**, **e**, **f**) chicken ceca stained with either rabbit anti-EtMIF, 1:500 (**a**, **c**, **d**, **e**), or control pre-bleed sera, 1:500 (**b**, **f**). **a**–**c** Ceca 24 h PI with Et. *D*, ceca from chickens infected with Et at 96 h PI. **e**, **f** Ceca from chickens infected with Et 120 h PI. *Arrowhead* in **e** points to wall-forming bodies of developing Ea oocysts

but, like in the present study, MIF appeared distributed in the parasite's cytoplasm and not specifically associated with an organelle. Consistent with our present observation, previous studies have shown that MIF is a leaderless protein that is secreted from cells by specialized, nonclassical pathways, and this may result in a more general distribution of MIF within

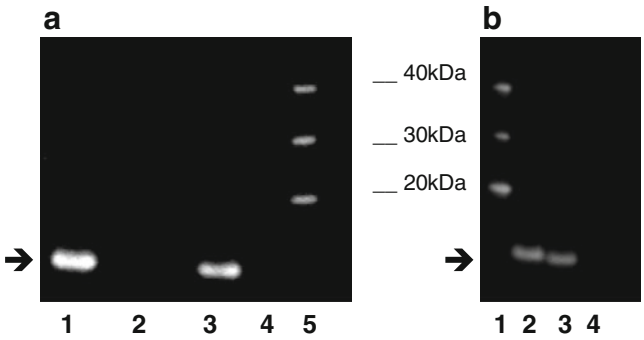

Fig. 4 Western blots showing that rabbit anti-rEtMIF and rabbit anti EaMIF lack reactivity to rChMIF. **a** *Lanes 1* and *3*, 100 ng rEtMF; *lanes 2* and *4*, 100 ng rChMIF; *lane 4* molecular weight standards. *Lanes 1* and *2*, primary antibody rabbit anti-rEtMIF 1:250; *lanes 3* and *4*, primary antibody rabbit anti-rEtMIF 1:500. **b** *Lane 1*, molecular weight standards; *lane 2*, 100 ng rEaMIF; *lane 3*, 100 ng rEtMIF; *lane 4*, 100 ng rChMIF. *Lanes 1–4*, primary antibody rabbit anti EaMIF 1:1,000

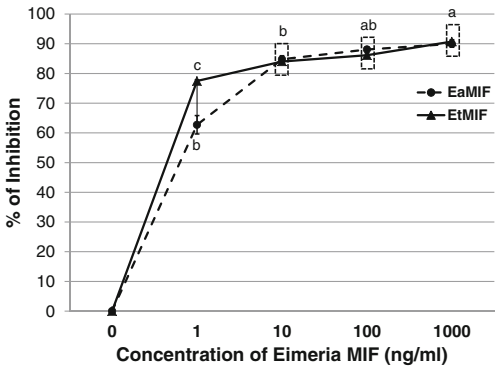

Fig. 5 Chemotaxis assay in modified 48-well Boyden chambers. Migration of adherent PBMCs was examined in the presence of different concentrations of EaMIF (10^{-3}, 10^{-2}, 10^{-1}, and 10^{0} μg/ml). Isolated adherent PBMCs were placed into the top chamber well separated by 5-μm pore membrane from *Eimeria* MIF in the bottom wells. The chemotaxis chamber was incubated for 4 h at 39 °C in humidified air containing 5 % CO_2. The cells were stained with DF staining method, and the number of cells was counted. Each experiment was set in triplicate, and the results represented the mean of three individual experiments. *Solid line with rectangular markers* represents percentage of inhibition by EaMIF, while *dash line with empty circle markers* represents percentage of inhibition by EtMIF. Each *letter* indicates statistically significant difference ($p<0.05$)

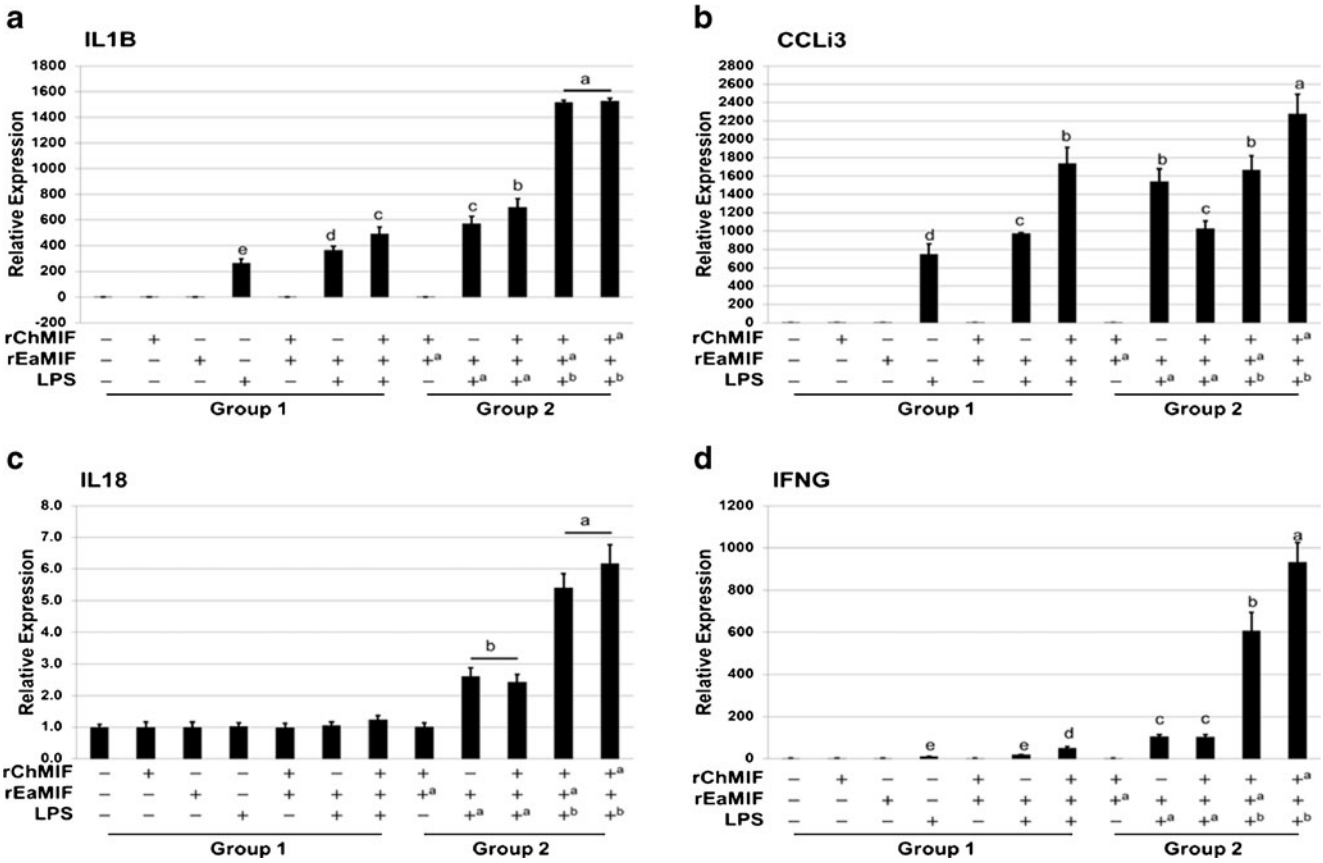

Fig. 6 Transcripts of pro-inflammatory cytokines and chemokines as influenced by EaMIF. Freshly cultured chicken adherent PBMCs were incubated for 6 h in two different groups as shown in Table 1. Cell lysis buffer was directly added into the wells and total RNA was extracted. qRT-PCR analysis to measure mRNA level of chicken IL-1B (**a**), CCLi3 (**b**), IL-18 (**c**), and IFNG (**d**). Each *letter* indicates statistically significant difference ($p<0.05$)

cells, and immunostaining of macrophages indicates MIF appears present throughout the cytoplasm and in the perinuclear area (Merk et al. 2009). MIF has also been previously shown to be associated with the nucleus and cytoplasm of tumor cells (Kamimura et al. 2000).

In order to effectively modulate host responses to infection of the invasive stages, the parasite should be able to excrete or somehow release MIF into the host environment. Using IHC to localize EtMIF in infected chicken ceca, immunoreactivity was observed as early as 24 h PI in the columnar epithelial cells that are also targets for infection by invading sporzoites. The staining was not co-localized with sporozoites, but it is possible that the staining recognizes MIF released by the parasite. An alternative explanation is that the observed staining was due to anti-EtMIF antibody cross-reacting with chicken MIF present in the ceca; however, Western blot analysis did not demonstrate reactivity of anti-EtMIF with rChMIF, suggesting that cross-reactivity was not present. Although MIF could not be co-localized with sporozoites, these stages of the parasite (schizonts, macorgmetes and oocysts) were clearly stained by anti-EtMIF and potentially release MIF into the surrounding chicken tissues. Miska et al. (2007) showed that EMIF is present in excretory/secretory products of Ea MZ, so it is quite likely that EMIF is secreted upon infection of the host. Additionally, cells of vertebrates secrete MIF when exposed to LPS (Calandra et al. 1994); therefore, the secretion mechanism of MIF may be conserved throughout.

The biological function of EMIF on chicken mononuclear cells was also examined. Similar to avian MIFs, both EaMIF and EtMIF showed inhibitory function of chicken macrophage migration. We could not observe a titratable decrease in inhibition by EMIF on chicken macrophage migration based on tested concentrations (1 to 1,000 ng/ml). At 1 ng/mL, 60–70 % inhibition was attained, and 90 % inhibition was attained at 1,000 ng/ml. Unlike EMIF, *Trichinella spiralis* MIF showed the highest inhibitory ability at 50 pg/ml, and its inhibitory ability was reduced at the higher concentration (Tan et al. 2001). A recent study by Jang et al. (2011) reported that ChMIF positively affected the migration of chicken macrophages, while EMIF did not have any effect on the same cell type. In our experiments, both EaMIF and EtMIF inhibited the migration of chicken monocytes. The primary difference between the two experimental protocols was that much lower

amounts of recombinant protein were used in the assay compared to 100 μg (the final concentration is unknown) used by Jang et al. (2011). We attempted to mimic physiological amount of MIF; however, it is possible that MIF may elicit different responses based on the amount of protein present.

As to the effect of EMIF on chicken mononuclear cells, EaMIF alone or together with ChMIF did not influence the expression of cytokines and chemokines in chicken adherent PBMCs. However, the combination of EaMIF (and/or ChMIF) and LPS led to enhanced production of pro-inflammatory immune elements. These results follow a similar pattern to those of ChMIF (Kim et al. 2010). Furthermore, sequential treatment of EaMIF and LPS showed greatest enhancement of inflammatory mediators at the mRNA levels than any other condition. These results indicate that EMIF may induce host anti-inflammatory cytokines by enhancing the expression of inflammatory mediators over a short-term period. On the other hand, EMIF itself may inhibit production of pro-inflammatory mediators. Kleemann et al. (2000) reported that the absence of MIF leads to activation of the transcription factor AP-1 via Jab1, resulting in activation of pro-inflammatory genes. Jab1 is inactivated in the presence of MIF, resulting in a negative impact on inflammation and cell growth (Kleemann et al. 2000). Thus, similar amino acid sequences and structure of EMIF with ChMIF may function as ChMIF to inhibit host inflammatory response and cell proliferation. Unfortunately, this hypothesis cannot explain why EaMIF enhances the production of pro-inflammatory cytokines and chemokines in the presence of LPS.

In summary, the results indicate that EMIF is detectable in host tissues as well as in the parasite following infection. EMIF may be secreted into surrounding host tissue; however, this aspect of EMIF has to be more closely studied. EMIF also inhibits migration of chicken monocytes in vitro and, therefore, may play a similar role in vivo. Additionally, the effect of EMIF on host mononuclear cells showed that it functionally enhances pro-inflammatory cytokines and chemokines in pre-stimulated monocytes. In summary, EMIF is a parasite protein that upon release may have an effect on the immune system of the host; however, the exact nature of this effect remains to be elucidated.

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